Engineered novel CD4 cis-dimer protein significantly enhances the binding of HIV-1 envelope glycoproteins

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Abstract

The CD4 molecule on the T-cell surface can present as monomer and dimer forms. Dimeric CD4 can be important for T-cell activation. As the primary receptor of HIV, the interaction between CD4 and HIV envelope glycoprotein (Env) plays a critical role in mediating HIV infection. Env binds to the CD4 ectodomain (ECD) which contains 4 Ig-like subdomains (4D). To better mimic the native dimer conformation and quaternary structure, we designed a novel soluble CD4 cis-dimer with ECD-4D fused to a dimer motif at the C-terminus. The CD4 dimer protein was expressed/purified from HEK293T cells and evaluated by multiple tests. CD4 dimer binding potency to Env proteins from various HIV-1 subtypes increased on average by >10 fold as measured by ELISA and had stronger binding affinity and broader dynamic range as measured by BLI, compared to the CD4 monomer protein. The CD4 dimer also bound well to the CD4 binding site (CD4bs) resurfaced core RSC3. The gp120-D368R CD4bs mutation diminished the binding to the CD4 dimer. The CD4 dimer could also bind to a CD4-specific therapeutic mAb and IgG in patient samples, with better activities compared to the monomer. AlphaFold 2 predicted CD4 cis-dimer structure is well-superimposed with known CD4 crystal structures. These findings imply that the soluble CD4 cis-dimer protein is in the desirable conformation, resulting in an increased binding to HIV-1 Env. This novel CD4 dimer can be a very useful molecule for HIV and immunology research.

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Materials and Methods

Recombinant proteins: CD4 homodimer His-tag (Conigen Bioscience, CSP-24004); CD4 monomer; HIV-1 envelope glycoproteins (Env) from subtype B (IIIB, JR-FL, AC10.29), BC (CN54), C (93ZM651, C1086) and AE (CM235, 93TH975), resurfaced core RSC3 and RSC3-G367R, and CD4-binding site(CD4bs) mutant AC10.29. Human CD4 UniProt# A0A4Y5UGE4.
SDS-PAGE: SDS-PAGE with reducing and non-reducing conditions were used to detect the dimer protein and its purity relative to monomer species.
Antibody binding ELISA: CD4 proteins were coated on 96-well microtiter ELISA plates (2 µg/ml) and detected by CD4-specific antibody and HIV-1 infected autoimmune patent IgG samples at a serial dilution.
CD4/Env binding measured by ELISA: CD4 protein dimer or monomer was coated on 96-well microtiter plate (2 µg/ml) to bind to HIV-1 Env proteins with serial dilutions, then the Env protein bound on CD4 was detected by anti-V3 rabbit monoclonal antibody.
CD4/Env binding measured by BLI: The dissociation constant (Kd) was determined using a Gator Pilot bio-layer interferometry (BLI) instrument. The CD4 dimer or monomer protein was immobilized on nickel probe and interacted with serial dilutions of HIV-1 Env proteins in a binding kinetics assay.
Computational modeling: Structures were predicted using the ColabFold implementation of AlphaFold2. All predictions were run using PDB100 template mode, MMseqs2 (Uniref + environmental), AlphaFold2 multimer v3 model, and 12 recycles. Crystal structure studies (Xtal) were retrieved from the RCSB-PDB, and all alignments and visualizations were created with PyMOL3.

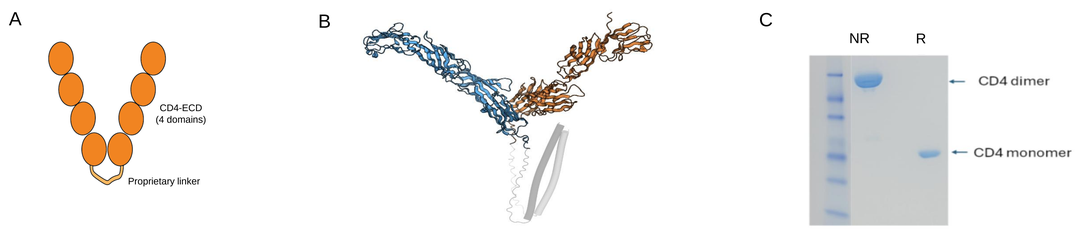


Fig. 1. CD4 homodimer protein design and expression. (A) The 4 domain ectodomain of human CD4 was genetically fused to a proprietary linker and dimerization motif to promote soluble dimer formation. The recombinant protein was expressed in HEK293T cells and purified. (B) Predicted structure of the recombinant CD4 homodimer agrees with Xtal. (C) SDS-PAGE analysis of the purified CD4 homodimer under non-reducing (NR) and reducing (R) conditions.

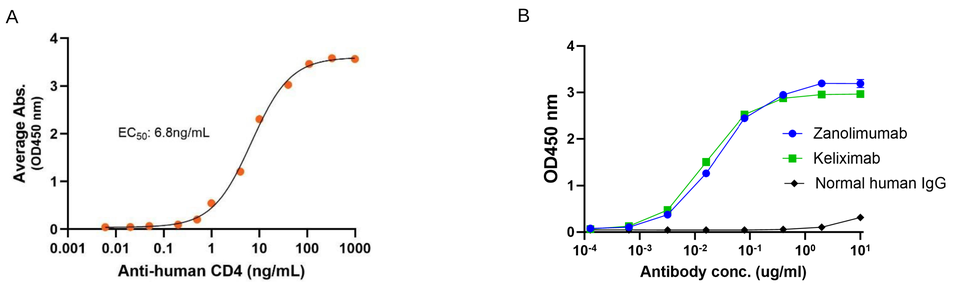
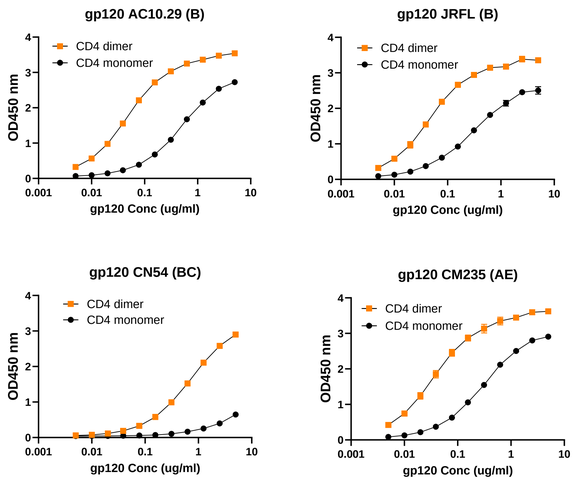


Fig. 2. CD4 protein dimer potently binding to CD4-specific monoclonal antibodies. (A) Binding to mouse mAb SIM.2 recognizing the same epitope as the Leu 3a antibody, blocking HIV-induced syncytium formation. (B) Binding to therapeutic human mAbs Zanolimumab (immunosuppressive drug, EC50 25 ng/mL) and Keliximab (treatment for severe chronic asthma, EC50 16ng/ml).



gp120 (subtype)	EC50 (mg/mL)		EC50 Monomer/ Dimer Ratio
	CD4 monomer	CD4 dimer	
AC10.29 (B)	0.518	0.05	10
JRFL (B)	0.31	0.046	7
CM235 (AE)	0.309	0.038	8
CN54 (BC)	29.56	0.734	40

Fig. 3. CD4 dimer or monomer protein binding to HIV-1 gp120 proteins from subtype B (AC10.29, JR-FL), BC (CN54) and AE (CM235). The CD4 dimer protein binding to gp120 protein increased 7-40 fold, respectively compared with CD4 monomer.

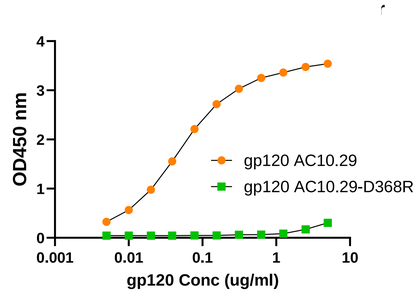
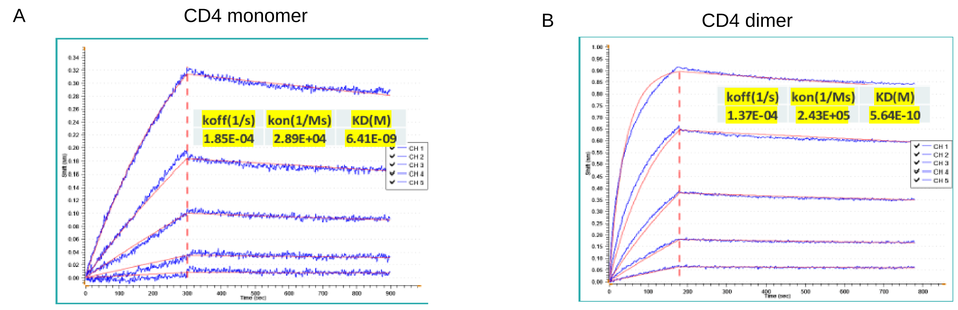


Fig. 4. HIV-1 gp120 protein CD4 binding site (CD4bs) mutation diminished the binding to CD4 dimer protein.



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HIV-1 Env protein (subtype)	koff(1/s)	kon(1/Ms)	KD (M)
gp120-IIIB (B)	1.94E-04	1.05E+05	1.85E-09
gp140-JRFL-CF (B)	1.92E-05	9.96E+04	1.92E-10
gp120-96ZM651 (C)	2.49E-04	2.63E+04	9.44E-09
gp140-C1086 (C)	<1.00E-006	1.09E+05	<1E-12
gp120-CM235 (AE)	7.26E-04	6.84E+04	1.06E-08
gp120-93TH975 (AE)	1.37E-04	2.43E+05	5.64E-10
RSC3	1.22E-04	6.75E+04	1.81E-09
RSC3-G367R	2.03E-04	3.17E+04	6.42E-09

Fig. 5. Using Label Free BLI Assay to evaluate CD4 protein monomer (A) and dimer (B) binding to gp120-93TH975 (AE) with 11-fold increase of binding affinity. (C) Using CD4 protein dimer for BLI assay (Gator Bio) to evaluate the CD4 receptor binding activities of HIV-1 envelope glycoproteins from various subtypes

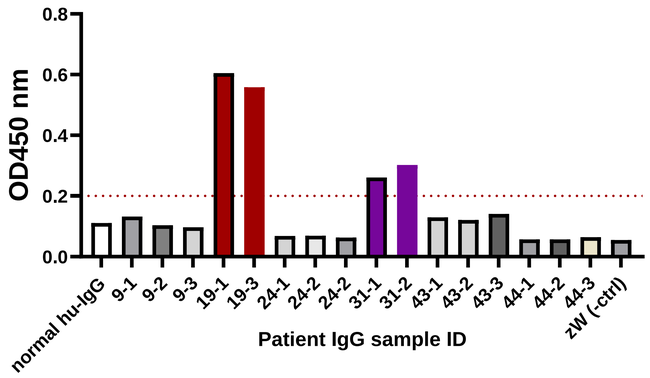

Fig. 6. Using Conigen's CD4 cis-dimer protein, CD4-specific antibodies were clearly detected in 2 patient IgG samples: red bars (19-1, 19-3), and purple bars (31-1, 31-2), that were not able to be detected using CD4 monomer protein.

Conclusions

- The recombinant CD4 4-domain homodimer is designed to mimic the native dimer structures as predicted.
- The CD4 protein dimer expressed from HEK293T cells is purified with high purity and demonstrated specific potent binding to the CD4-specific functional antibodies.
- The CD4 protein dimer significantly increased the binding potencies to the HIV-1 envelope glycoproteins compared to monomer and presents the correct conformation.
- The CD4 protein dimer has demonstrated better binding to CD4-specific antibodies in autoimmune patient IgG samples.
- The novel 4-domain CD4 dimer can be potentially very useful for HIV and immunology research and drug discovery.

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